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(71) Applicant *(for all designated States except US)*: SENSOR-CHEM INTERNATIONAL CORPORATION [CA/CA]; 170 College Street, Room 308A, Toronto, Ontario M5S 3E3 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: MCGOVERN, Mark [CA/CA]; 170 College Street, Room 308A, Toronto, Ontario M5S 3E3 (CA). THOMPSON, Michael [CA/CA]; 170 College Street, Room 308A, Toronto, Ontario M5S 3H6 (CA).

(74) Agent: HELLER, David, J.; Ridout & Maybee, 19th floor, 150 Metcalfe Street, Ottawa, Ontario K2P 1P1 (CA).

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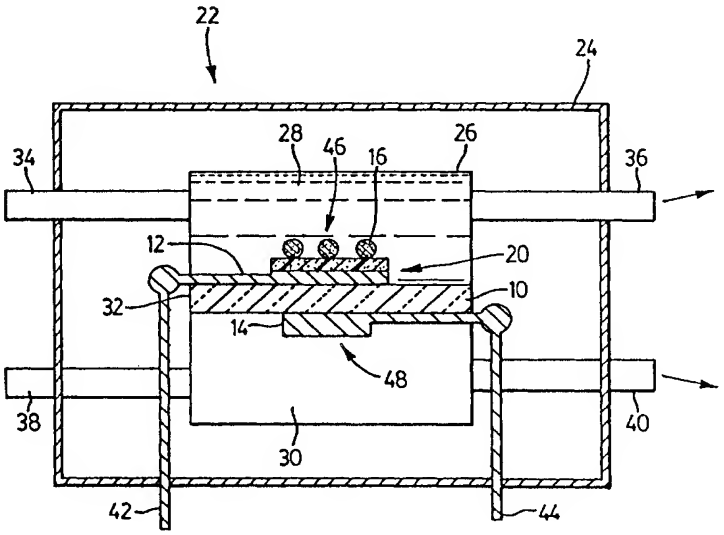

(57) Abstract

An apparatus (22) is provided for monitoring small molecule–biomolecule interactions. An oscillating substrate (10) has a wet surface (46) for contact with a liquid and for binding with a biomolecule and a dry surface (48). A detection apparatus (42, 44) determines the resonance frequency of said substrate before and after interaction. Thus, small molecules are monitored for interaction with biomolecules by contacting a solution containing the small molecule with immobilized biomolecules under test, and generating acoustic waves using an acoustic wave device. Interaction of the small molecules with the immobilized biomolecules causes a change in frequency, which can be detected and analyzed electronically to monitor the interaction.

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TITLE OF THE INVENTION

APPARATUS AND PROCESS FOR MONITORING AND DETECTING SMALL MOLECULE - BIOMOLECULE INTERACTIONS

FIELD OF THE INVENTION

This invention relates to methods of and apparatus for qualitative and quantitative analysis of biomolecule interactions using viscosity measurements.

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority from Canadian patent application serial number 2,271,179, filed May 5, 1999.

BACKGROUND OF THE INVENTION

The principle of a biosensor is to transform biologically interesting phenomena, such as the binding of a drug molecule with a target biomolecule, into electronic information which can be more readily accessed and processed. A typical biosensor comprises a

biochemical component attached to a form of electronic transducer. The biochemical component, usually comprising proteins or nucleic acids, is exposed to a particular chemical compound, and any resulting chemical interaction is electronically detected by the transducer.

Transducer technology for use in biosensors can be based on piezoelectric effects, which are changes in shape or conformation of certain solid crystals when an electric voltage is applied to them, or, conversely, the production of an electric voltage when such a solid crystal is mechanically deformed. If the crystal is used as one of the components of an "oscillating circuit", the crystal will determine the oscillation frequency of the whole circuit. The crystal itself vibrates at a "resonant frequency" which is determined by the physical shape and size of the crystal, among other factors. Quartz is the most commonly used piezoelectric crystal, although many others exist. Under suitable conditions, the circuit will oscillate very accurately at the same frequency, which is measured in Hertz (Hz).

Piezoelectric crystals can be used as the basis of biosensor transducer platform technologies. If any material is allowed to contact a clean piezoelectric crystal surface, as the device oscillates, it will change the resonant frequency of the device. The size of the observed frequency change can be used to measure the quantity of material which adhered to the crystal surface.

These devices have been used to analyze liquid samples for the presence and content of macromolecular biochemical substances such as nucleic acids by hybridization thereof to a complementary nucleic acid immobilized on a quartz crystal forming part of a piezoelectric circuit. In such an arrangement, a biosensor transducer platform comprising a platform-like

quartz crystal, a first electrode on its lower surface and a second electrode on its upper surface, with the immobilized biomolecule on the second, upper electrode, is used. The resulting bonding or hybridization of the nucleic acid in the test solution (analyte) to the immobilized nucleic acid on the electrode causes a change in the vibrational frequency of the circuit, as compared with that of the circuit involving the immobilized nucleic acid itself. The existence and magnitude of the change of frequency is a measure of the presence and quantity of the nucleic acid under test, and can be electronically translated into detection of the presence and measurements of the quantity of the nucleic acid under test, in the analyte solution.

A mathematical expression known as the Sauerbrey equation has been developed, to describe the piezoelectric effects of substances bound to the piezoelectric crystal. This predicts that an increase in mass of the substance bound to the piezoelectric crystal will cause a proportional change in the frequency of oscillation of the circuit. It also indicates that, to measure mass changes in a meaningful way, the change must be of the order of at least one nanogram (one billionth of a gram), so that the method would only be useful for measurement of macromolecules.

United States patent No.5,595,908 to Fawcett teaches a method for detecting polynucleotide hybridization using a piezoelectric crystal. A polynucleotide is immobilized on a surface of a piezoelectric crystal. After washing and drying the crystal, the resonance frequency of the piezoelectric crystal is measured through means for determining the resonant frequency of a piezoelectric crystal. A separate source of polynucleotide is exposed to the polynucleotide-coated piezoelectric crystal for a sufficient length of time and under

conditions suitable for hybridization. After washing and drying the crystal, the resonance frequency of the crystal is then again measured, and the difference between the resonance frequency before and after the incubation period indicates the extent of hybridization. The washing and drying steps required by Fawcett's method render it expensive and time consuming.

United States patent No. 5,478,756 to Gizeli, et al. teaches a chemical sensor which includes a piezoelectric support capable of supporting a shear horizontal wave provided on its surface with an electrode, for detecting antibody-antigen binding reactions. Gizeli et al. do not provide details regarding the use of their chemical sensor.

Neither Fawcett nor Gizeli et al. teach a method for monitoring the binding of small molecules to biomolecules.

There is an ongoing need for improved methods for detecting and monitoring small molecules. This arises, for example, in the screening of drug candidates, which for the most part are "small molecules", for activity or binding affinity with certain target molecules.

SUMMARY OF THE INVENTION

The invention provides a method for monitoring small molecule-biomolecule interactions, comprising the steps of:

(a) binding a biomolecule to a substrate;

(b) contacting the biomolecule with a liquid;

(c) inducing shear oscillation of the substrate;

(d) determining frequency of oscillation of the acoustic wave device to obtain a first value;

(e) introducing a small molecule into the liquid;

(f) subsequently measuring frequency of oscillation of the acoustic wave device to obtain a second value; and

(g) comparing the first value to the second value.

In one embodiment, the substrate is a part of an acoustic wave device, and the acoustic wave device is selected from the group consisting of a piezoelectric device, a magnetic acoustic resonator sensor, a surface acoustic wave device, a thin rod acoustic wave device, a shear horizontal acoustic wave device, and a plate mode with acoustic sensor.

In an embodiment, the biomolecule is bound to the electrode, and the electrode is bound to the acoustic wave device. The biomolecule may be bound to the electrode by physiosorption or by binding using neutravin-biotin, thiol-gold or TTU silane.

The biomolecule may be a polynucleotide, a polypeptide, and it may be any biological molecule or compound which undergoes a conformational change upon binding to a small molecule.

In an embodiment, the liquid is aqueous. The liquid may contain a physiological buffer.

The method of the invention may further comprise the subsequent steps of:

(h) removing the liquid by introducing new liquid not containing the small molecule;

(i) measuring frequency of oscillation of the acoustic wave device to obtain a third value; and

(j) comparing the third value to the second value.

The method of the invention may also further comprise the subsequent steps of:

(k) introducing into the new liquid a second small molecule;

(i) measuring frequency of oscillation of the acoustic wave device to obtain a fourth value;

(j) comparing the fourth value to the third value.

In one embodiment, the small molecule is less than 2800 daltons. In another embodiment, the molecule is less than 1000 daltons. In a further embodiment, the small molecule is less than 500 daltons. The small molecule may be a pharmaceutical agent.

In an embodiment, the method of the invention further comprises the step of contacting a side of the acoustic wave device with a gas. The gas may flow across a side of the substrate. The gas side may be maintained separate from the liquid side.

The invention also provides an apparatus (22) for monitoring small molecule-biomolecule interactions, comprising:

(i) an oscillating substrate (10);

(ii) a wet surface (46) attached to the substrate for contact with a liquid and for binding with a biomolecule;

(iii) a dry surface (48) attached to the substrate; and

(iv) a detection apparatus (42, 44) for determining the resonance frequency of the substrate.

In an embodiment, the apparatus may further comprise a liquid flow chamber (28) for flowing liquid over the wet surface. In an embodiment, the apparatus further comprises a gas flow chamber (30) for flowing gas over the dry surface.

In various embodiments of the apparatus of the invention, the substrate and the detection apparatus together are an acoustic wave device selected from the group consisting of a piezoelectric sensor, a magnetic acoustic resonator sensor, a surface acoustic wave device, a thin rod acoustic wave device, a shear horizontal acoustic wave device, and a plate mode with acoustic sensor.

In one embodiment, the biomolecule is bound to an electrode (12), and the

electrode is bound to the detection apparatus. The biomolecule may be bound to the electrode by physiosorption or by binding using neutravin-biotin, thiol-gold or TTU silane.

In one embodiment, the wet surface is located on a face of the substrate opposing the dry surface.

Thus, according to the present invention, in one aspect, there is provided a process for detecting the interaction of small molecules with biomolecules, which comprises contacting a liquid solution suspected of containing a small molecule of interest with the biomolecules in a biosensor, the biosensor comprising a piezoelectric material, an electrode electrically connected to the piezoelectric material, the biomolecules immobilized on the electrode, and an electrical circuit involving the electrode and the piezoelectric material and having characteristic, measurable electrical output signals, and monitoring change in at least one of the electrical output signals caused by interaction of the small molecule of interest with the immobilized biomolecules.

BRIEF DESCRIPTION OF THE DRAWINGS

A specific embodiment of the present invention is diagrammatically illustrated in the accompanying drawings, in which:

FIGURE 1 is a diagrammatic top view of a piezoelectric sensor platform for

use in the invention;

FIGURE 2 is a diagrammatic side view thereof;

FIGURE 3 is a diagrammatic side view of the top electrode of the device with biomolecules immobilized thereon;

FIGURE 4 is a similar view of the biosensor mounted in a flow cell.

FIGURE 5 is a schematic diagram of a DNA sequence bound to a biosensor.

FIGURE 6 is a graph showing frequency modulation in response to the addition of small molecules to the flow cell.

FIGURE 7 is a graph showing frequency modulation in response to the addition of small molecules to the flow cell (DNA-antibiotic interaction).

FIGURE 8 is a graph showing frequency modulation in response to the addition of small molecules to the flow cell (RNA-antibiotic interaction).

FIGURE 9 is a graph showing frequency modulation in response to the addition of small molecules to the flow cell (RNA-peptide interaction).

DESCRIPTION OF EMBODIMENTS

The present invention provides a process whereby biosensors based upon viscosity effects and resonance measurements as described herein may be used to detect, to quantify and to monitor the chemical and biochemical reactivity and properties of small molecules, i.e. those of less than about 2,800 Da molecular weight. Contrary to the theory and predictions derived from the Sauerbrey equation, frequency changes caused by binding of small molecules to biomolecules such as nucleic acids

and proteins immobilized on oscillating circuits much larger than was expected can be produced, and, in fact, in the opposite direction from that predicted. For example, the piezoelectric crystal-based device when operated with the piezoelectric crystal in contact with liquid is effectively much more sensitive than the Sauerbrey equation suggests. It can effectively measure adhered matter in the picogram (one thousandth of one billionth of a gram, 10^{-9} gram) range, making it useful in detection of the adherence of small molecules to immobilized biomolecules such as proteins and nucleic acids in such piezoelectric biosensors.

It has been found, according to the invention, that acoustic wave devices (AWDs)contacting a liquid on at least one surface no longer obey the Sauerbrey equation. This equation now appears to hold true only for AWDs operating in gases or in vacuum. In a liquid medium, an AWD undergoes a dramatically fundamental change in the manner it operates and responds to adhered matter. In fact, the vibrating AWD transmits ultrasonic or acoustic waves into the liquid medium. While it is not intended that this invention should be limited to any particular theory of operation, it appears that the liquid closest to the AWD surface can "slip" along the vibrating surface, so that it is the viscosity and "stiffness" of the liquid which is being probed by the ultrasonic or acoustic waves emitted by the vibrating AWD. In a manner which may be reminiscent of sonar, the transmitted acoustic waves may couple back to the AWD, and thereby detect the presence of, and hence changes in the characteristics of, nearby molecules. The molecules in fact change the frequency of the acoustic waves. Binding of or interaction of small molecules with biomolecules immobilized on or near the AWD causes further changes, as compared with the immobilized biomolecules

themselves.

The challenge of detecting small molecules using acoustic waves is well known in the art. The literature associated with the monitoring of changes in frequency due to molecular binding invariably invokes a mass response model. As discussed above, the Sauerbrey equation is used to correlate the observed frequency change with the quantity of mass which adheres to the crystal surface. As shown in Table 1, application of the Sauerbrey equation to methods and apparatus known in the prior art, in this case, a piezoelectric biosensor, predicts that small drug molecules, formula weights of approximately 500 to 600, would result in insignificant changes in resonance frequency, on the order of 0.1 Hz. Given that the background noise is typically at least 1.0 Hz, the methods and apparatus known in the prior art was completely ineffective in identifying small molecule interaction uses. Table 1 then lists actual change in frequency observed using the methods and apparatus of the present invention. These frequency changes are readily observable.

Table 1 - Challenge of Detecting Small Molecule Interactions

Compound	**Formula Weight**	**ΔF as Predicted by Sauerbrey Equation[a]**	**Actual ΔF Measured (maximum observed)**
Neomycin	617 g/mol	-0.11 Hz	23 Hz
Streptamycin	478 g/mol	-0.09 Hz	7 Hz
Gentamycin	580 g/mol	-0.11 Hz	7 Hz
Tat_{12}	1617 g/mol	-0.30 Hz	30 Hz

Since the sensors described herein operate on a principle of transmitting

ultrasonic waves in a liquid medium, they are termed "acoustic wave biosensors", or acoustic wave devices, AWDs, and this term is sometimes used herein to denote such devices. Other acoustic wave devices which can be used in the present invention, besides piezoelectric devices, include magnetic acoustic resonator sensors (MARS), surface acoustic wave devices, thin rod acoustic wave devices, shear horizontal acoustic wave devices, and plate modes with acoustic sensor. Examples of piezoelectric crystals suitable for the present invention include quartz, lithium tantalate, Rochelle salt, and lead titanate zirconate ceramics

Where a piezoelectric crystal is used, means for detecting the resonance frequency of a crystal can be provided in a variety of ways. Generally, a crystal will be interposed between electrode material, the leads of which are connected to an oscillator circuit. A frequency meter or the like attached to the output of the oscillator circuit is used to measure the resonance of the crystal.

The electrode material need not be in physical contact with the crystal. However, an efficient way of preparing a piezoelectric crystal for use in the present invention is to deposit electrode material on opposite surfaces of the crystal. For example, on a typical quartz crystal, the electrode material can be deposited on the central region of opposing crystal surfaces. In addition, a band of electrode material can be deposited on the crystal surface to form a pathway from the electrode material deposited on the central region of crystal surface to the edge of the crystal where leads for connecting to an oscillator circuit can be attached.

Any suitable electrode material can be used in the practice of this invention. Such materials include, but are not limited to, gold, silver, aluminum, nickel, chromium, titanium tantalum and the like.

It will be appreciated that not all AWDs require an electrode to measure frequency. For example, where the AWD is a magnetic acoustic resonator sensor, typically, the glass wafer upon which the biomolecule is bound will be in contact with a frequency counter.

Biomolecules can be chemically bonded directly to a piezoelectric crystal surface or indirectly via the electrode or via a material previously deposited on the crystal surface. Such other material can be electrode material or a thin layer of polymeric or other bonding material, such as intermediate molecule links. For example, biomolecules may be bound to the electrode by physiosorption or by binding using neutravin-biotin, thiol-gold or TTU silane. As a particular example, a synthetic polynucleotide can be bonded chemically to the crystal surface to which a second, naturally occurring, polynucleotide can be attached by enzymatic ligation. More particularly, a polynucleotide can be ligated by enzymatic reaction (DNA or RNA ligase) to a different polynucleotide which is covalently bonded by chemical reaction to a functional group which is in turn chemically bonded to a substrate, for example, a polymeric substrate.

Effective polymeric substrates include those polymers characterized by hydrolytically stable, hydrophobic backbones substituted with reactive pendant groups.

For example, such polymers include, but are not limited to, copolymers of ethylene or propylene and N-(6-x-hexyl)-acrylamide, copolymers of styrene and p-x-methyl styrene, copolymers of alkylated siloxanes and 6-x-hexyl and alkyl substituted siloxanes, and similar polymers where x is a reactive group such as amino, sulfhydryl, iodo, bromo, chloro, carboxyl, hydroxy, chloro carbonyl, dimethylsilyl and similar groups which are capable of combining with polynucleotides or derivatized polynucleotides. Other polymers useful in the present invention include, but are not limited to, poly(butyl methacrylate), polyurethane and the like.

One of the most sensitive areas for immobilizing the polynucleotides is the central portion of the crystal surface.

In an embodiment of the process of the invention, the liquid solution suspected of containing the small molecule of interest is flowed continuously across the immobilized biomolecules, and measurements of chosen electrical signals are made continuously as the solution flows. A variety of different solutions can be flowed across the biomolecules of the device successively, in a continuous operation, and measurements correlated to the different solutions. Thus, for example, subsequent to taking readings with respect to a first small molecule's interaction with the biomolecule, one may introduce new liquid not containing that small molecule, and measure the frequency of oscillation of the acoustic wave device to determine whether the small molecule remains bound to the biomolecule, and if so, for how long. Subsequently, one may introduce liquid carrying a second small molecule, and measure the frequency of oscillation to obtain data regarding the binding of that

molecule. In this way, screening of a number of small molecules, e.g. drug candidates, for interaction with biomolecules can be conducted rapidly, reliably and economically. As used herein, biomolecules includes proteins and nucleic acids, and other polypeptides and polynucleotides, as well as any biological molecule or compound which may undergo a change in size, shape, or conformation upon binding with a small molecule.

In an embodiment of the invention, the AWD is not completely immersed in the liquid. By only immersing part of the AWD, for example, only the surface attached to the biomolecule, the vibration of the AWD is less damped and produces better data, and the risk of shorting out the electrode is reduced.

The electrical signal used as the basis of measurement of changes caused by the interaction can be any detectable output which changes as a result of the interaction. For example, it can be the frequency of oscillation of the piezoelectric crystal, as detected by the circuitry. Preferably, however, changes in impedance of the crystal are used as the basis of measurement. For this, pulsed electrical power is supplied to the crystal, and the resolution of the impedance measurements can be improved by using a selection of different frequencies of the power input.

A specific type of piezoelectric-based AWD for use in the present invention is a thickness shear mode device, TSM.

One process of the present invention accordingly uses an acoustic wave device

(AWD), nucleic acids immobilized on the AWD, a flow cell which contains the AWD and which permits the flow of liquids across the surface of the AWD to which the biomolecules are attached, a means of sending and receiving electronic signals to and from the AWD to determine changes in acoustic frequency associated with small molecule interaction with nucleic acid or protein targets, a means of storing and processing the electronic signals collected from the AWD, and a method to interpret the data and correlate it to the determination of small molecule interaction affinity for biomolecules.

As noted above, the AWD is a type of biosensor used as a means of detecting the presence of molecules dissolved in a liquid medium. An AWD produces and propagates acoustic waves into a liquid medium.

Thus from one aspect, the present invention provides a process, and suitable apparatus, for detecting or monitoring the interaction of small molecules, especially those of molecular weight 2800 Da or less, with biomolecules such as nucleic acids, which comprises immersing immobilized biomolecules under test in a solution containing the small molecule of interest, generating acoustic waves in the solution by use of an AWD, and detecting and analyzing frequency changes in the acoustic waves attributable to interaction of the small molecules of interest with the immobilized biomolecules under test.

Typically, the AWD is made of a piezoelectric material, such as quartz, and is shaped into planar form, often circular. The device should also be shaped in such a

way as to allow the surfaces to vibrate parallel to the plane of each face. To each face, metal electrodes are affixed to allow intimate electrical contact, so that the piezoelectric effect can occur.

The AWD is made useful for biosensor applications by attaching or immobilizing biomolecules onto the AWD surface. It is well known that biomolecules interact very selectively with other molecules to form aggregate compounds. By immobilizing a particular biomolecule species onto the AWD, a very selective biosensing device can be made.

There are many immobilization protocols described in the literature. In particular, silane adhesion agents have been used to attach biomolecules to biosensors, including piezoelectric AWD's. One specific and highly effective method is disclosed in International Patent Application PCT/CA/00969.

By attaching one end of the biomolecule which is not involved in small molecule interaction to the surface of the AWD, the remaining portions of the biomolecule are free to associate with small molecules dissolved in liquid.

In one embodiment, the AWD is housed in a flow cell which performs several functions. It protects the AWD from damage. It allows electrical contact to be made with the AWD, and allows the electronic signals to pass from the AWD, through the flow cell, and to the outside of the flow cell, where the contacts terminate. It also allows liquid or gas to flow over one or both sides of the AWD. Each face of the

AWD is suitably positioned over a separate chamber of a pre-determined volume. The liquid flows into one of the chambers through one port, through the chamber, and out of the chamber through a second port. The other chamber is not connected to the liquid supply, and may be kept sealed or purged with gas. The faces of the AWD are sealed, typically by using "o-rings".

Liquids can be introduced into the flow cell by means of a suitable pump, such as peristaltic, syringe, or piston, so that a continuous flow of liquid passes through the flow cell. Water is the most commonly used liquid for this purpose; however, many additives may be dissolved in the water so as to provide an environment suitable for measuring biomolecular interactions. Cations (such as lithium, sodium, potassium, magnesium, calcium, ammonium, alkyl ammonium, quaternary ammonium, guanidinium), anions (such a chloride, phosphate, carboxylate, sulfate, sulfonate, carbonate, borate), buffers (to regulate pH), solubilizing agents (detergents, surfactants, organic solvents), chelators (such as EDTA), and anti-bacterial/anti-microbial substances may be present in the water.

Gases can be introduced through the flow cell, if desired, by means of a pressurized tank, so that continuous flow of gas passes through the flow cell. Air may be used for this purpose, however, almost any inert gas will be suitable. In one embodiment, the gas used is relatively dry, in order to maintain a constant humidity in the gas chamber of the flow cell. Alternatively, the gas chamber may be kept at a constant humidity by means of effective sealing of the chamber.

In most typical pharmaceutical drug screening applications, the present invention may be used in conjunction with multiple samples of small molecules passing into the flow cell to evaluate their affinities for the biomolecule immobilized onto the AWD. The small molecules are normally stored in separate containers, or can also be stored as mixtures. The sample concentrations can be either known or unknown. A known volume of sample is injected into the flow cell for analysis by means, for example, of a Rheodyne sample injection valve. One method is to use a commercially-available "autoinjector" device which possesses such a valve, and is capable of injecting known volumes of sample into the continuously flowing liquid, which then travels through the appropriate tubing to the flow cell. Each sample is injected sequentially. The autoinjector method allows multiple samples to be processed in a predetermined order automatically. The autosampler "XXL 232" supplied by Gilson is suitable for this purpose.

To send and receive electronic signals, the electrical contacts, which terminate on the outside of the flow cell, are connected to an appropriate electronic measurement device which is capable of reading the particular frequency that the AWD is operating, at a given interval of time. To do this, the electronic measurement device should be capable of transmitting electrical power to the AWD, as well as being capable of reading frequency. One such method is known as the "network analysis method", in which a Hewlett-Packard 4395A network/spectrum/impedance analyzer is used to characterize the AWD primarily by what is known as "series resonant frequency". Many other parameters such as parallel resonant frequency, phase, impedance, resistances, capacitances, and inductances may be used, in an analogous manner. The

Hewlett-Packard 4395A is controlled by a computer program, which may be installed on a separate computer system, that allows the measurements to be started at a predetermined time and date, carried out at predetermined intervals of time throughout the course of an experiment, and stopped at a predetermined time and date.

The frequencies, and other electronic parameters, that are detected by the Hewlett-Packard 4395A, are also stored as a data file in an appropriate format which allows the data to be graphed as time vs frequency, or time vs some other electronic parameter. This allows the magnitude and/or the area of the peaks present in the data graph to be determined.

The magnitude and/or the area of the peaks over time correspond to the relative strength of the small molecule interaction. If one small molecule sample generates a greater peak height, and/or peak area signal compared to a signal generated by a different small molecule, then the first small molecule can be interpreted as having a greater affinity for the biomolecule than does the second small molecule. If both small molecule samples generate the same signal intensity over time, but the first sample was known to be more dilute than the second sample, then the first sample can be interpreted as having a greater affinity for the biomolecule than does the second small molecule.

Such a procedure for data analysis can be carried out automatically using commercially available software such as those typically used to process chromatographic data.

Figs. 1 and 2 show a quartz substrate 10 having a top electrode 12 on its top surface and a similar bottom electrode 14 on its bottom surface, both in electrical contact with the substrate. The arrows on Fig. 2 indicate the ability of the substrate to oscillate in the plane of its surfaces on application of electric power of appropriate frequencies.

Fig. 3 shows biomolecules 16, e.g. nucleic acids or proteins, immobilized to the upper surface of the top electrode 12 through the intermediary of a chemical immobilizing agent 18, e.g. a cross-linked silane optionally including linkers and tethers as disclosed in aforementioned International Patent Application PCT/CA98/00969, the disclosure of which is incorporated herein by reference.

Fig. 4 shows the biosensor 20, including the substrate 10, electrodes 12, 14 and immobilized biomolecules 16 inside a flow cell 22 and ready for operation in the process of the invention. The flow cell 22 has an outer housing 24 inside which is mounted a cell 26 having an upper chamber 28 and a lower chamber 30. The biosensor 20 is mounted on a seal 32 separating the chambers 28 and 30, with the top electrode 12 and the immobilized biomolecules 16 protruding into the upper chamber 28 and the bottom electrode 14 protruding into the lower chamber 30. The biosensor has a wet surface 46 attached to the substrate 10 for contact with a liquid and for binding with a biomolecule. On the opposing side of substrate 10 there is a dry surface 48. Liquid containing the small molecule of interest fills the upper chamber 28 and flows continuously therethrough, from liquid inlet 34 to liquid outlet 36, both protruding

outside the housing 24. Gas such as nitrogen fills the lower chamber 30, and flows continuously therethrough from gas inlet 38 to gas outlet 40, similarly protruding outside the housing 24. The use of inert gas in this manner permits free oscillation of the piezoelectric substrate, and maintains an inert environment, of controlled humidity (preferably dry) in contact with the bottom surface and bottom electrode14, for increased reliability of results.

In operation, liquid inlet 34 is connected via suitable pumping arrangements to a multiwell plate containing a plurality of different liquid solutions for analysis. The solutions are pumped sequentially through the upper chamber 28 of the flow cell, while the electrodes of the substrate are connected to suitable circuitry via electrical connections 42, 44. Readings of output from the electrodes are made and suitably displayed continuously, in real time, as the solutions are flowed through, and appropriately recorded for analysis.

Small molecules of molecular weight up to about 2,800 Da are monitored for biomolecule interactions according to the present invention. One specific example of such a molecule is the Tat-20 peptide, which interacts with RNA (TAR) which can be immobilized as the biomolecule in the present invention. This provides a monitor of HIV infection.

The method of the invention can be used to screen the activity of small molecules of up to about 2800 Da molecular weight, for their interaction with various nucleic acids immobilized on the substrate. As used herein, the term "small molecule"

refers to a molecule with a molecular weight of less than 2800 daltons. In another embodiment, "small molecule" refers to a molecule with a molecular weight of less than 1000 daltons. In yet another embodiment, "small molecule" refers to a molecule with a molecular weight of less than 500 daltons. In an embodiment, the small molecule is a potential pharmaceutical agent, such as a potential diagnostic agent or drug.

Screening of drug candidates for such interactions is increasingly important in research and development, in the pursuit of active small molecules capable of, for example, inhibiting the activity of viral RNA and other nucleic acids, such as those found in HIV infected patients. A particular class of small molecules with which the process of the invention has been used with notable success is the class of antibiotics known as aminoglycosides, which includes such well-known antibiotics as streptomycin, neomycin and gentamycin. These are highly charged molecules, which interact with nucleic acids. Accordingly the method of the invention is suitable for screening these and other compounds of the same general family for their interaction with specific nucleic acids. Another specific example of application of the present invention is in connection with the toxicity of drug compounds, in regard to unwanted nucleic acid binding, which may be minimized by use of the process of the present invention with such compounds. Other uses of the invention include determining under what conditions a small molecule will not bind to a given biomolecule, obtaining information regarding changes in tertiary structure of biomolecules, measuring affignity, potency, specificity, on/off (complexing/ separating) rates, and other pharmacokinetic and metabolic studies.

EXAMPLES

Materials:

Piezoelectric crystals were rinsed with ACS-grade acetone, ACS-grade ethanol and copious amount of ddH_2O, followed by drying with a stream of dry helium, prior to any immobilization. All buffers / solutions were degassed with dry helium prior to use.

1. Tris buffer (pH 7.5): 10 mM Tris-HCl
   0.2 mM EDTA
   70 mM NaCl
   pH adjustment with NaOH
2. PBS buffer (pH 7.4): 10 mM phosphate
   2.7 mM KCl
   137 mM NaCl
3. PBS buffer (pH 3.0): same as above, pH adjustment with HCl
4. DNA: unless otherwise indicated, all DNA solutions are made in degassed Tris buffer (pH 7.5) and all strands are 25 base-pairs long.

Example 1 - DNA hybridization LOD determination (Neutravidin-based)

A 9 MHz gold (Au) piezoelectric quartz crystal was washed, dried and mounted in the flow cell. Tris buffer was passed through the flow cell at pump speed #1 (~ 0.065 mL/min) and the baseline is monitored until stable. 475 uL of neutravidin

(NA), 1mg/mL in Tris, was injected into the circuit at the same flow rate. This plug was allowed to pass over the crystal in a flow-through manner. At the lowest flow rate, the residence time is approximately 7 minutes, which proves to be adequate for most types of immobilization. The change in frequency was allowed to stabilize prior to the next injection and this same allowance is made for all subsequent steps. 300 uL of 30 uM biotinylated ssDNA (b-F1) was injected over the NA surface, also at the same flow rate. Solutions of the complementary strand (F2) of increasing concentrations (475 uL of 100 nM, 1 uM, 5 uM, 10 uM and 20 uM) are injected successively over the immobilized b-F1 surface. Changes in the resonant frequency are observed for the 100 nM and 1 uM solutions, after which the surface is fully saturated.

Example 2 - DNA hybridization LOD determination (HDI-based)

A 9 MHz chromium (Cr) piezoelectric quartz crystal was washed and placed in a humidifying chamber overnight. Silanisation was performed in a glovebox, via immersion in a 1 mM solution of TTU in dried toluene, for a period of 2 hrs. The crystal was then rinsed with dried toluene, ACS-grade chloroform and dried with a stream of nitrogen. A 20 uM solution of thiol-terminated ssDNA in PBS (pH 7.4), was reacted with the linker bis-bromomethyl benzene-2-sulfonate (BMBS) for 1 hr. The resulting complex was then purified in a NAP column, conditioned with PBS pH 3 to minimize analyte retention. The purified aliquot was pH-adjusted to 7.5, introduced onto the TTU surface and allowed to incubate for 1 hr. The crystal was then rinsed thoroughly with ddH_2O, dried with a stream of helium and mounted in the flow cell.

Tris buffer was passed through the flow cell at pump speed #1 (~ 0.065 mL/min) and the baseline was monitored until stable. Solutions of the complementary strand (F2) of increasing concentrations (475 uL of 100 nM, 1 uM, 5 uM, 10 uM, 20 uM) were injected successively over the immobilized b-F1 surface. Changes in the resonant frequency were observed for all five solutions, after which the surface is fully saturated.

Example 3 - Preparation of double-stranded DNA surfaces for assays

The procedure outlined in Examples 1 and 2 was used, with the exception being that in the last step, only one 50 uM solution of F2 was used to ensure the maximum degree of hybridization. Figure 6 shows a schematic diagram of a DNA sequence, SCDNA102, which has known binding sites for various antibiotics, bound to a biosensor.

Example 4 - Small molecules interaction with prepared surfaces using ssDNA biomolecule

Crystals prepared using the methods set out above were mounted in the flow cell and the appropriate buffer is passed through to establish a baseline. Crystals could be modified just prior to assay and kept in the flow cell under Tris until the appropriate buffer switch is performed. The analytes were injected at various volumes (475 uL - 200 uL) and flow rates (pump speed #1 - #5), according to the nature of the interaction and the desired throughput. Higher flow rates will produce sharp, chromatographic-

like peaks and allows for higher throughputs but at pump speed higher than #5 (~ 0.3 mL/min), the peristaltic pump introduces unacceptable baseline variations (noise). The analytical concentration for each type of assay varies according to the strength of the interaction but is generally in the high nM to the low uM range.

Figure 6 is a graph showing frequency modulation in response to the addition of small molecules to the flow cell to assess the interaction of the small molecules with single stranded SCDNA102, shown in Figure 5. At separate time intervals, various antibiotics were added to the flow-through system, using the following pattern: small molecule A, wash, small molecule A, wash, small molecule B, wash, small molecule B, wash. As is seen from Figure 6, actinomycin, daunomycin, ethidium bromide, and echinomycin all produced marked, reproducible peaks or valleys in the frequency data. In contrast, the application of distamycin and tetracycline failed to produce any noticeable effect. This is to be expected as SCDNA102 does not contain distamycin and tetracycline binding sites (see Figure 5).

The results of this experiment is tabulated in Tables 2 and 3. As can be seen in these tables, the method provides not only information regarding the presence/ absence of a change in frequency, but also provides data concerning the area and width of the peak or valley, which can reveal valuable information about the biomolecule-small molecule interaction. Having regard to Table 3, the reliability of the present invention is demonstrated with the low standard deviation as between paired trials.

Table 2 - ssDNA results: SCDNA102

drug	area	width	height
actinomycin (10uM)	2861.3	3.36	14.2
daunomycin (10uM)	1011.7	2.49	6.79
distamycin (100um)	1177.75	2.81	7.14
echinomycin (1uM)	3462	4.46	12.94
Ethidium Bromide (100uM)	4173.4	3.76	18.54
tetracycline (100uM)	0	0.00	0

Table 3 - ssDNA results: SCDNA102

drug	result#1	result#2	average	SD
actinomycin (10uM)	14.25	14.15	14.20	0.07
daunomycin (10uM)	6.33	7.25	6.79	0.65
distamycin (100um)	8	6.27	7.14	1.22
echinomycin (1uM)	12.5	13.38	12.94	0.62
Ethidium Bromide (100uM)	17.93	19.14	18.54	0.86
tetracycline (100uM)	0	0	0.00	0.00

Example 5 - Drug small molecules interaction with prepared surfaces using RNA biomolecule

Using the methodology and apparatus set out above, the interaction between neomycin and immobolized TAR RNA was assessed. Neutravidin-biotin was used as the tether to bind the RNA sequence to the substrate, and varying concentrations of neomycin were passed, at intervals, through the flow cell. As shown by the graph at Figure 8, neomycin created clear peaks in the frequency, and there does appear to be some relation between concentration of neomycin and the size of the peak. Results of a similar run are tabulated in Table 4. Results of another similar run are found at Figure 7.

Table 4 - TAR - Neomycin

drug	[uM]	height
neomycin	1	7.28
neomycin	10	8.43
neomycin	100	18.74
neomycin	1000	41.89
neomycin	10000	41.14

Results for a similar experiment with TAR_{31} and various antibiotics small molecules are set out in Table 5.

Table 5 - Direct Detection of Antibiotics Against TAR_{31}-RNA Target Immobilized[1] on Acoustic Wave Biosensor

	Experimental Results		
Drug	**Concentration**	**Signal Intensity (Hz)**	**Published IC_{50}***
Neomycin	1 μM	3 +/- 1	0.92 μM
Gentamycin	10 μM	2 +/- 1	45 μM
Streptomycin	100 μM	5 +/- 1	9.5 μM
Other Cmpds			
Tat_{12}	200 nM	7 +/- 1	-

Example 6 - Confirmation of small molecules interaction with prepared surfaces using control

Using the methodology and apparatus set out above, the interaction between various antibiotics and the prepared flow cell, absent bound biomolecule, was assessed. No biomolecule was bound to the substrate, and varying concentrations of various antibiotic were passed. at intervals, through the flow cell. As shown by Table 6, no

signal was produced in this control experiment.

Table 6 - Control Experiments to Test for Non-Specific Interactions[1] in the Absence of TAR_{31}-RNA

	Control Results	
Drug	**Concentration**	**Signal Intensity (Hz)**
Neomycin	1 μM	0
Neomycin	10 μM	0
Gentamycin	10 μM	0
Gentamycin	100 μM	0
Streptomycin	100 μM	0
Streptomycin	1000 μM	0

Example 7 - Peptide small molecules interaction with prepared surface using RNA biomolecule

Using the methodology and apparatus set out above, the interaction between the HIV related peptide Tat-20 and immobolized TAR RNA was assessed. Injections of Tat-20 were passed, at intervals, through the flow cell. As shown by the graph at Figure 9, Tat-20 created clear peaks in the frequency measured. Results from a similar experiment are also noted in Table 5.

Although embodiments of the invention have been disclosed for illustrative purposes, it will be appreciated that variations or modifications of the disclosed apparatus lie within the scope of the present embodiments.

CLAIMS

We claim:

1. A method for monitoring small molecule-biomolecule interactions, comprising the steps of:
(a) binding a biomolecule to a substrate;
(b) contacting said biomolecule with a liquid;
(c) inducing shear oscillation of said substrate;
(d) determining frequency of oscillation of said acoustic wave device to obtain a first value;
(e) introducing a small molecule into said liquid;
(f) subsequently measuring frequency of oscillation of said acoustic wave device to obtain a second value; and
(g) comparing said first value to said second value.

2. A method as claimed in claim 1, wherein said substrate is a part of an acoustic wave device, said acoustic wave device selected from the group consisting of a piezoelectric device, a magnetic acoustic resonator sensor, a surface acoustic wave device, a thin rod acoustic wave device, a shear horizontal acoustic wave device, and a plate mode with acoustic sensor.

3. A method as claimed in claim 2, wherein said acoustic wave device is a piezoelectric device.

4. A method as claimed in claim 3, wherein said substrate is quartz.

5. A method as claimed in any one of claims 3 or 4, wherein frequency of oscillation is determined by measuring electrical output of said acoustic wave device.

6. A method as claimed in claim 5, wherein said biomolecule is bound to said

electrode, and said electrode is bound to said acoustic wave device.

7. A method as claimed in claim 6, wherein said biomolecule is bound to said electrode by physiosorption or by binding using neutravin-biotin, thiol-gold or TTU silane.

8. A method as claimed in claim 7, wherein said biomolecule is bound to said electrode by binding using neutravin-biotin.

9. A method as claimed in claim 7, wherein said biomolecule is bound to said electrode by binding TTU silane.

10. A method as claimed in any one of claims 1-9, wherein said biomolecule is a polynucleotide.

11. A method as claimed in claim 10, wherein said biomolecule is DNA.

12. A method as claimed in claim 10, wherein said biomolecule is RNA.

13. A method as claimed in any one of claims 1-9, wherein said biomolecule is a polypeptide.

14. A method as claimed in claim 13, wherein said biomolecule undergoes a conformational change upon binding to a small molecule.

15. A method as claimed in claim 1, wherein said liquid is aqueous.

16. A method as claimed in claim 16, wherein said liquid is contains a physiological buffer.

17. A method as claimed in claim 1, further comprising the subsequent steps of: (h) removing said liquid by introducing new liquid not containing said small

molecule;

(i) measuring frequency of oscillation of said acoustic wave device to obtain a third value; and

(j) comparing said third value to said second value.

18. A method as claimed in claim 17, further comprising the subsequent steps of:

(k) introducing into said new liquid a second small molecule;

(i) measuring frequency of oscillation of said acoustic wave device to obtain a fourth value; and

(j) comparing said fourth value to said third value.

19. A method as claimed in claim 10, wherein said small molecule is less than 2800 daltons.

20. A method as claimed in claim 10, wherein said small molecule is less than 1000 daltons.

21. A method as claimed in claim 10, wherein said small molecule is less than 500 daltons.

22. A method as claimed in claim 10, wherein said small molecule is a potential pharmaceutical agent.

23. A method as claimed in any one of claims 1-22, further comprising the step of contacting a side of said acoustic wave device with a gas.

24. A method as claimed in claim 23, wherein said gas flows across said side.

25. A method as claimed in claim 23, wherein said gas side is maintained separate from said liquid.

26. An apparatus (22) for monitoring small molecule-biomolecule interactions,

comprising:

(i) an oscillating substrate (10);

(ii) a wet surface (46) attached to said substrate for contact with a liquid and for binding with a biomolecule;

(iii) a dry surface (48) attached to said substrate; and

(iv) a detection apparatus (42, 44) for determining the resonance frequency of said substrate.

27. An apparatus as claimed in claim 26, further comprising a liquid flow chamber (28) for flowing liquid over said wet surface.

28. An apparatus as claimed in claim 26 or 27, further comprising a gas flow chamber (30) for flowing gas over said dry surface.

29. An apparatus as claimed in claim 26, wherein said substrate and said detection apparatus together are an acoustic wave device selected from the group consisting of a piezoelectric sensor, a magnetic acoustic resonator sensor, a surface acoustic wave device, a thin rod acoustic wave device, a shear horizontal acoustic wave device, and a plate mode with acoustic sensor.

30. An apparatus as claimed in claim 29, wherein said acoustic wave device includes a piezoelectric material.

31. An apparatus as claimed in claim 30, wherein said piezoelectric material is quartz.

32. An apparatus as claimed in claim 30 or 31, wherein a biomolecule is bound to an electrode (12), and said electrode is bound to said detection apparatus.

33. An apparatus as claimed in claim 32, wherein said biomolecule is bound to said electrode by physiosorption or by binding using neutravin-biotin, thiol-gold or TTU silane.

34. An apparatus as claimed in claim 33, wherein said biomolecule is bound to said electrode by binding using neutravin-biotin.

35. A apparatus as claimed in claim 33, wherein said biomolecule is bound to said electrode by binding using TTU silane.

36. A apparatus as claimed in any one of claims 26 to 35, wherein said wet surface is located on a face of said substrate opposing said dry surface.

37. A apparatus as claimed in any one of claims 26 to 36, wherein said apparatus is located within a flow cell.

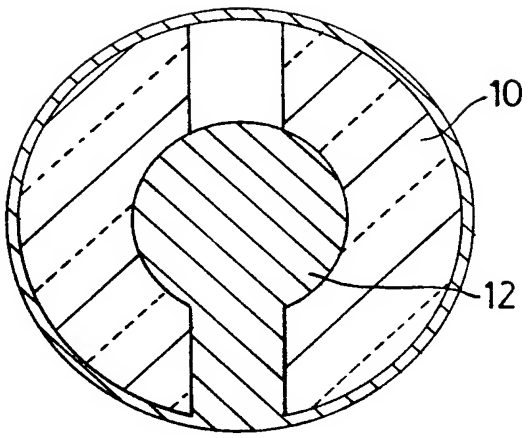


FIG. 1

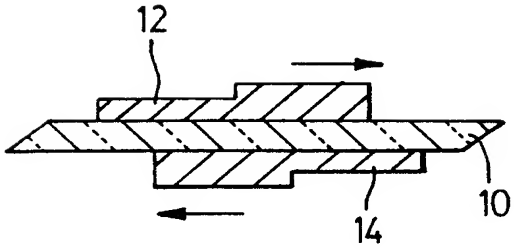


FIG. 2

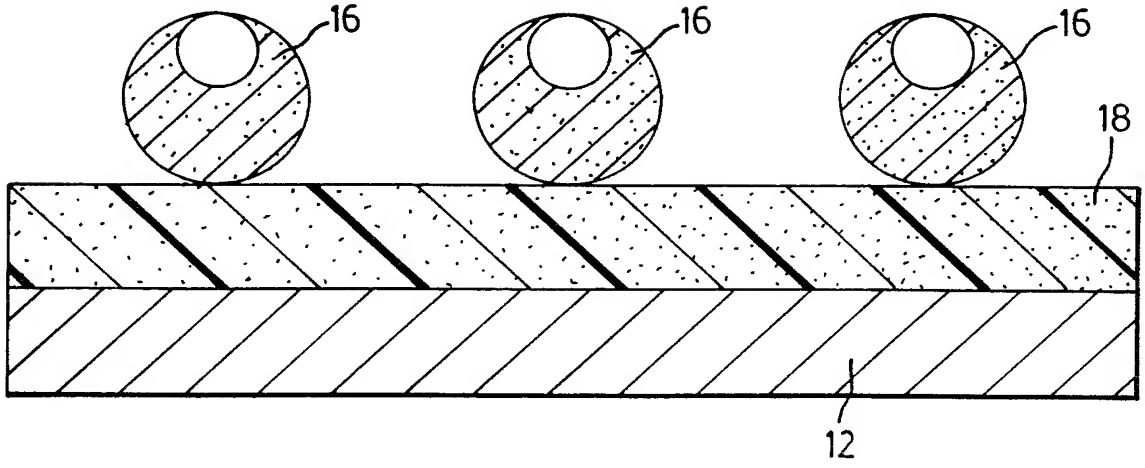


FIG. 3

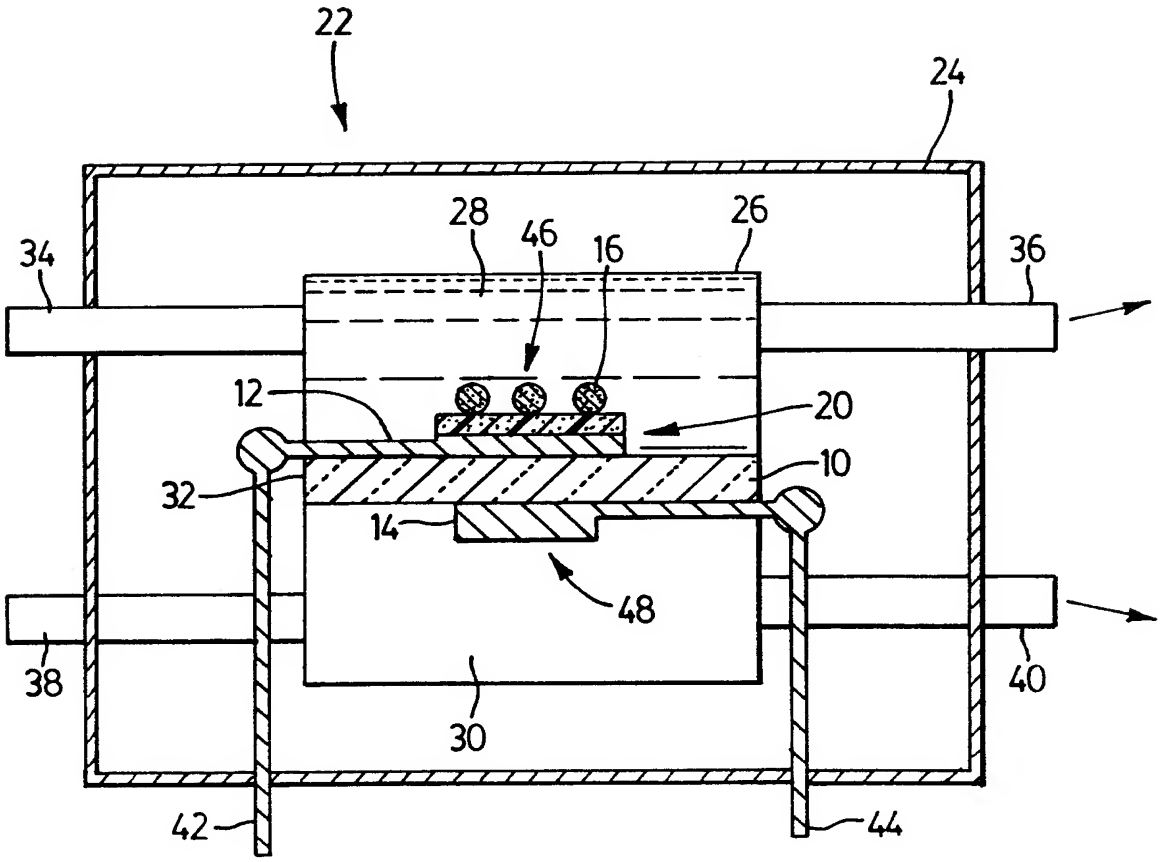


FIG. 4

Fig. 5 - Schematic of the "SCDNA102" DNA Sequence and Known Drug Binding Sites

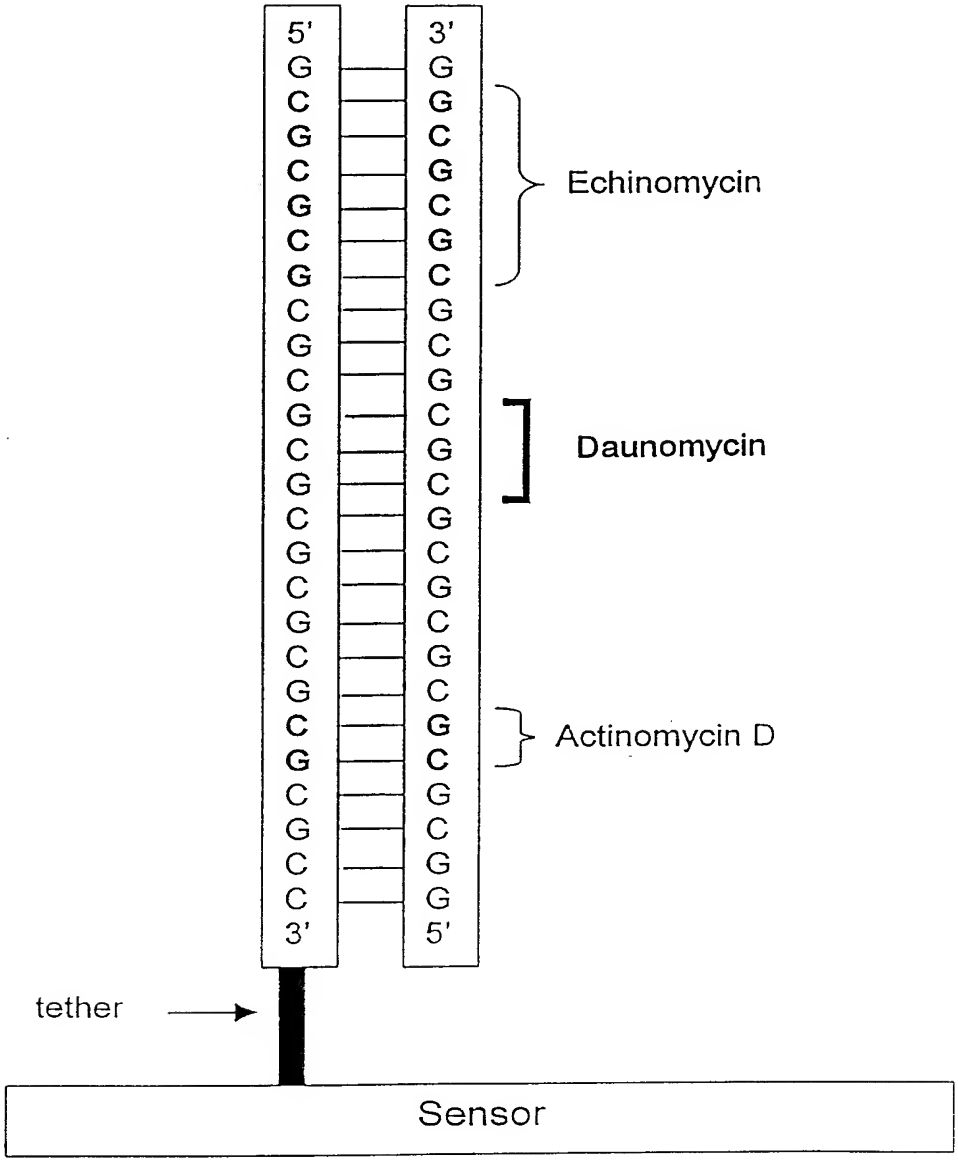


Fig. 6 - Biosensor Data: Raw Output of Antibiotics Interacting with Immobilized ssDNA in Flow-Through System

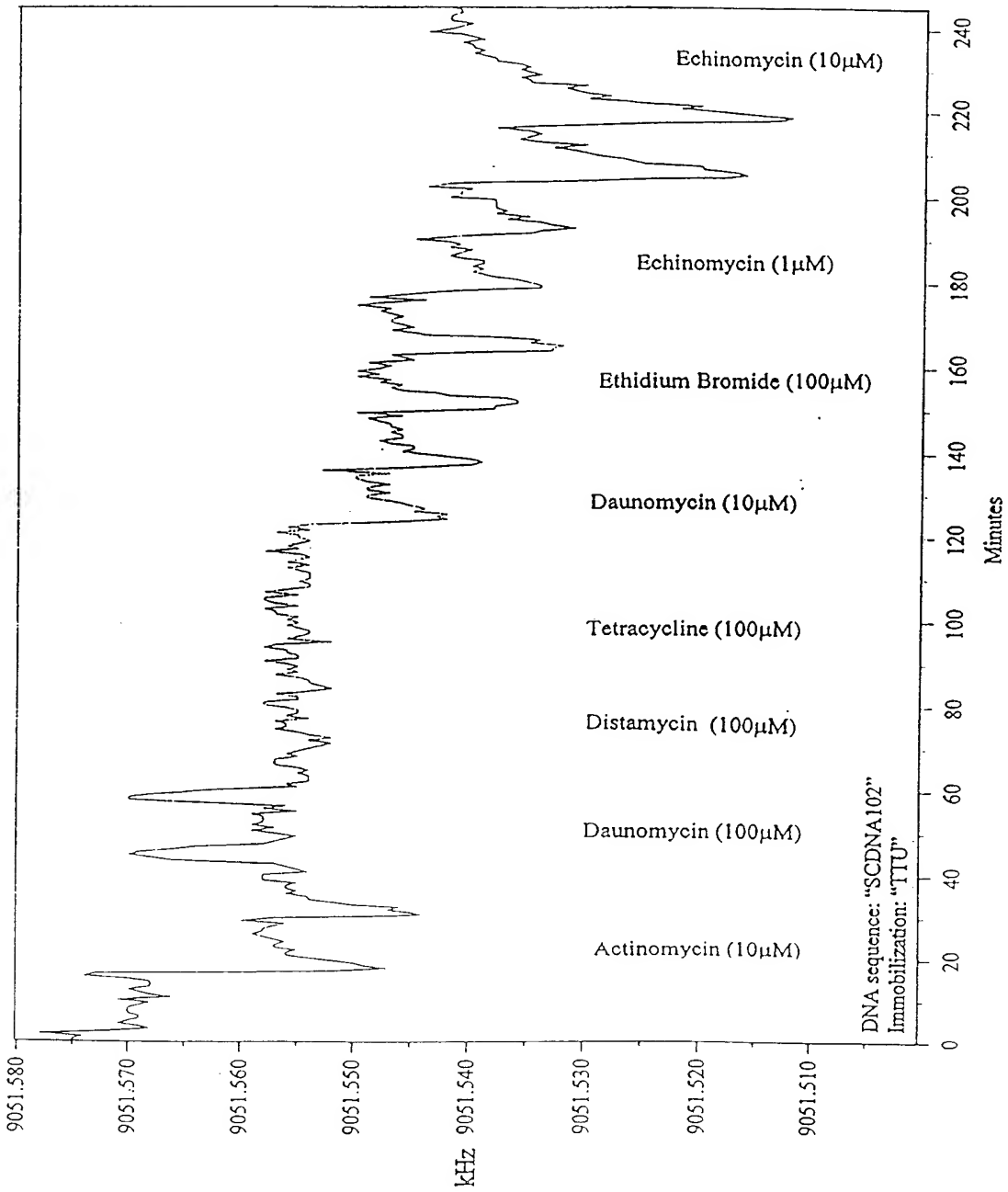


Fig. 7 - Biosensor Data: Antibiotic (Neomycin) Interacting with Immobilized RNA ("TAR")

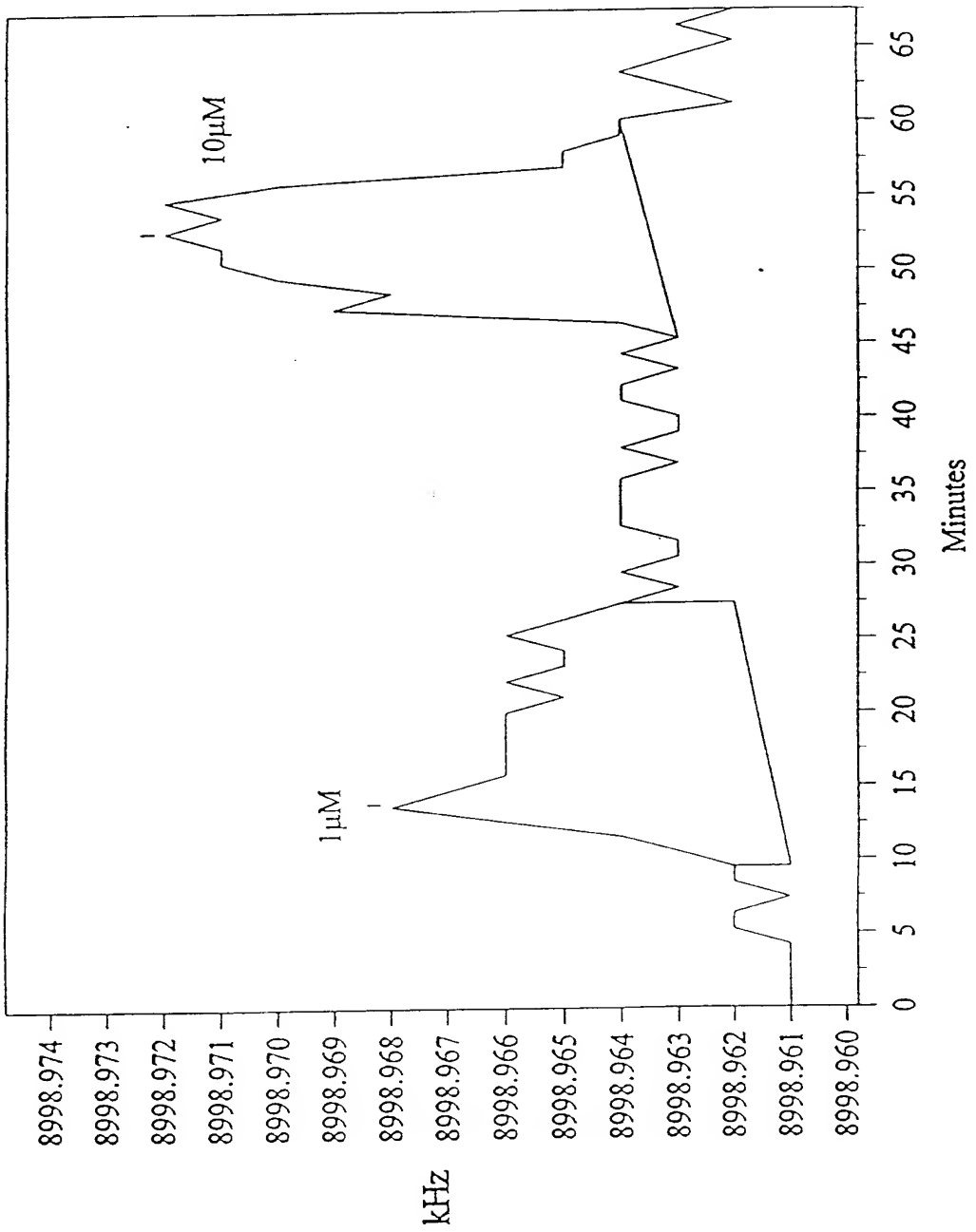
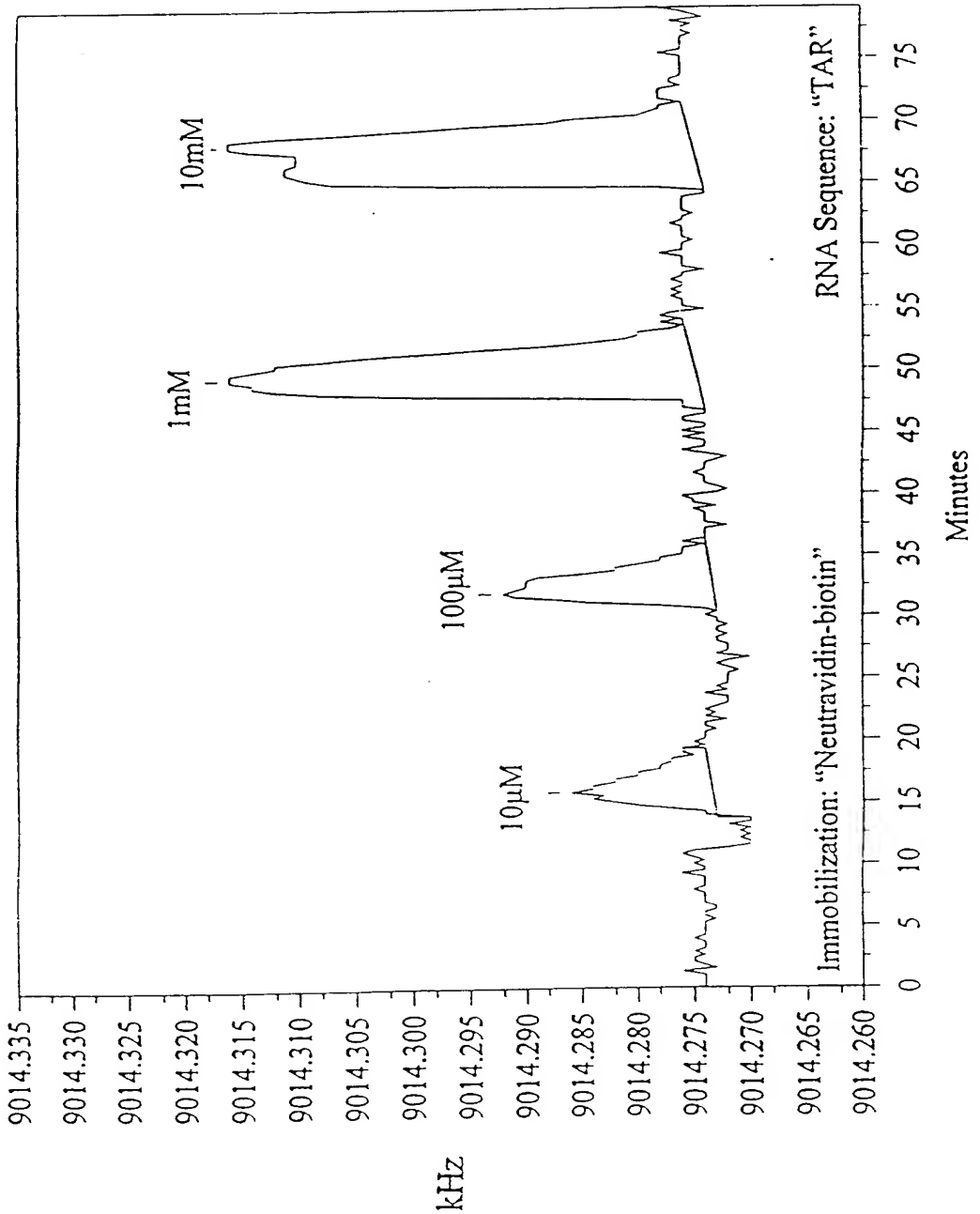


Fig. 8 - Biosensor Data: Antibiotic (Neomycin) Interacting with Immobilized RNA ("TAR")



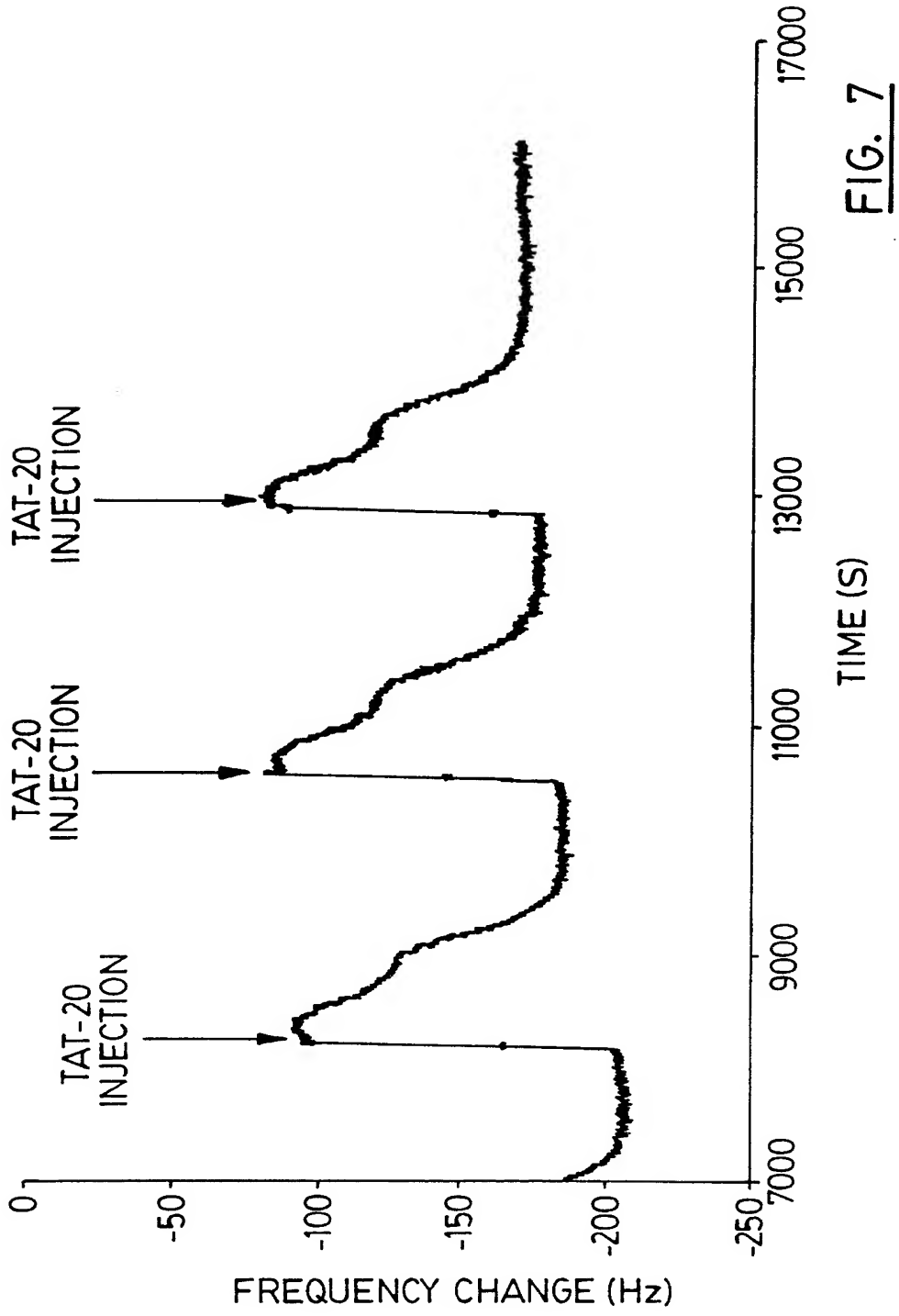

TAT-20 INJECTION
TAT-20 INJECTION
TAT-20 INJECTION
0
-50
-100
-150
-200
-250
FREQUENCY CHANGE (Hz)
7000
9000
11000
13000
15000
17000
TIME (S)

FIG. 7